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Geno- and seroprevalence of *Felis domesticus* Papillomavirus type 2 (FdPV2) in healthy cats

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Abstract

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Geno- and seroprevalence of *Felis domesticus* Papillomavirus type 2 (FdPV2) in healthy cats

Papillomaviruses can cause proliferative skin lesions ranging from benign hyperplasia to squamous cell carcinoma (SCC). However, asymptomatic infection is also possible. Several groups have detected FdPV2 DNA in association with feline Bowenoid in situ SCC (BISC). Therefore, a causative connection has been suggested. However, only very little is known about the epidemiology of FdPV2. The aim of this study was to define its geno- and seroprevalence in healthy cats.
For this purpose an FdPV2-specific PCR assay was used to analyse Cytobrush samples collected from 100 healthy cats. Moreover, an ELISA was established to test the sera obtained from the same cats for antibodies against the major capsid protein (L1) of FdPV2.
The genoprevalence of FdPV2 amounted to 98%. Surprisingly, the quantities of viral DNA detected in the samples from the healthy cats exceeded the amounts detected in samples from feline BISC lesions. The seroprevalence was much lower, amounting to 22%. The concentrations of antibodies against FdPV2 were relatively low in healthy cats, whereas they were very high in cats with BISC.
These observations suggest that FdPV2 is highly prevalent, even among healthy cats. However, cats that carry it on their skin mount only rarely an antibody response. In contrast, cats with BISC show a strong antibody response. Together, these data imply that active replication of FdPV2 may precede the occurrence of BISC, while it is no longer needed for the progression of the disease.

Keywords: Cat, FdPV2, BISC, Papillomavirus, prevalence

Zusammenfassung

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Geno- und Seroprävalenz von *Felis domesticus* Papillomavirus Typ 2 (FdPV2) bei gesunden Katzen

Papillomaviren können proliferative Hautläsionen auslösen, welche von benignen Hyperplasien bis zu Plattenepithelkarzinomen reichen. Asymptomatische Infektionen sind jedoch auch möglich. Mehrere Studien konnten FdPV2 DNA im Zusammenhang mit felinen Bowenoiden in situ Karzinomen (BISC) nachweisen. Eine kausative Verbindung wurde daher vermutet. Über die Epidemiologie von FdPV2 ist jedoch nur wenig bekannt. Das Ziel dieser Studie war es, die Geno- und Seroprävalenz bei gesunden Katzen zu bestimmen.
Ein FdPV2-spezifischer PCR Assay wurde benutzt, um Cytobrush-Proben von 100 gesunden Katzen zu analysieren. Weiter wurde ein ELISA etabliert, um Serumproben derselben Katzen auf Antikörper zu testen gegen das Major Capsid Protein (L1) von FdPV2.
Die Genoprävalenz von FdPV2 lag bei 98%. Die Menge viraler DNA in Proben gesunder Katzen war dabei deutlich grösser als in Proben von felinen BISC-Läsionen. Die Seroprävalenz war viel tiefer und lag bei 22%. Die Konzentrationen an Antikörpern waren bei gesunden Katzen relativ tief während sie bei BISC-Katzen sehr hoch waren.
Diese Beobachtungen deuten darauf hin, dass FdPV2 auch unter gesunden Katzen weit verbreitet ist. Katzen, welche das Virus auf der Haut tragen, zeigen jedoch nur geringe Antikörperreaktionen. Dem gegenüber zeigen Katzen mit BISC hohe Antikörpertiter. Dies kann bedeuten, dass eine aktive Replikation von FdPV2 vor der Entwicklung von BISC stattfindet, während sie für das Fortschreiten der Erkrankung nicht weiter vonnöten ist.

Schlüsselwörter: Katze, FdPV2, BISC, Papillomavirus, Prävalenz

Introduction

Papillomaviruses (PV) are small DNA viruses. They possess a double-stranded, circular genome of approximately 8 kilobasepairs (kbp). The genome typically contains a long control region (LCR) and up to ten designated translational open reading frames (ORF) that are all transcribed from the same strand. According to their location on the genome, these ORFs can be divided into early (E1-E8) and late (L1-L2) ORFs representing the early and late region, respectively. The early regions encode viral regulatory proteins whereas the late regions encode the capsid proteins. The capsid is of icosahedral shape and consists of major capsid protein L1, organised in 72 pentameric subunits, and minor capsid protein L2. The virion is non-enveloped.[1]

PV are classified based on similarities and identities of the nucleotide sequence of their L1 ORF. Currently, more than 190 types of PV are described and categorised into 29 genera.[2,3] New PV types are continuously detected. PV can be found in various higher vertebrates including mammals, birds and reptiles.[1,2] About two third of the described PV are human specific while the remaining types are specific for a wide variety of animals.[2] Most host species are infected by multiple different PV species and types.[4–7]

Clinically, PV show a specific cellular tropism for squamous epithelial cells.[1] They can cause proliferative lesions ranging from benign warts to SCC.[1,2] However, asymptomatic infection seems to be even more frequent.[2] Up to 80% of people are infected asymptomatically with human PV.[4] Apart from a few exceptions, PV tend to be highly species specific.[1,8,9] Up to 2007, seven PV specific for *Felidae* were described. They were isolated from five different species, namely *Felis catus*, *Puma concolor* (Cougar), *Lynx rufus* (Bobcat), *Panthera leo* (Lion) and *Panthera unica* (Snow Leopard).[10,11] All feline PV characterized that time were classified into the genus *Lambdapapillomavirus.*[2] Interestingly, results from phylogenetic analysis proposed a coevolution of these viruses with their hosts.[12]

Meanwhile, four different specific PV could be discovered in the domestic cat alone.[2,3,13].The first sequences of the second feline PV were found in 2006.[14] After sequencing its whole genome in 2007, it was named feline PV type 2 (FdPV2) and classified into the newly created genus *dyo-Thetapapillomavirus.*[2,15] Although its 7899bp genome is comparable to other PV in size, only 6 ORFs could be identified. The early region comprises of E6, E7, E1 and E2 whereas the late region comprises of L2 and L1.[15]

Initially, FdPV2 DNA had been solely detected in feline Bowenoid in situ carcinomas (BISC). BISC is a rare premalignant state of squamous cell carcinoma (SCC).[6,16] Together with actinic keratosis, it belongs to the group of SCC in situ in felines.[16,17] Histologically, the neoplastic cells are limited to the epidermis leaving the basement membrane still intact.[16] BISC are non-painful, pigmented, plaque like lesions within the haired skin. They can occur at any site of the body and are usually multiple. In some cases they were reported as partially alopecic and covered by crusts.[6,16–18] The affected patients are typically older cats. No gender or breed predisposition could be

found. Surgical excision seems to be curative and no cases of metastasis have been reported so far. However, there are some reports of BISC that were left untreated and progressed to infiltrative SCC.[16,17]

Only little is known about the epidemiology of FdPV2 infections. A pre-existing infection with feline immunodeficiency virus (FIV) does not seem to be associated with higher FdPV2 infection rates.[19] Since its discovery, FdPV2 DNA has been found in BISC by various research groups with prevalences ranging from 18% to 100% (see table 6 in appendix). Besides, it was repeatedly amplified from viral plaques. Viral plaques are uncommon, non-neoplastic skin lesions that are clinically indistinguishable from BISC. Although complete regression has been reported, viral plaques are assumed to be precursor lesions of BISC.[20,21] All these studies support a causative role of FdPV2 in the development of viral plaques and BISC. However, most of these studies only include small numbers of cats. Furthermore, FdPV2 could also be found in other types of feline skin lesions. As in BISC, the determined prevalence rates of FdPV2 in these other lesions show a rather wide variety, comparable to those found in BISC lesions.[8,9,18,20,22–24] . Only few studies included samples from cat's normal skin. Amplification of PV DNA using broad range primers always failed. However, in one study, a set of specific primers was used. FdPV2 DNA could be amplified from 52% of the samples.[19]

There are no reports about the seroprevalence of FdPV2. Indeed, no assay for the measure of FdPV2 specific antibodies was developed so far. We therefore established a GST capture ELISA[25] for detection of antibodies directed against the major capsid protein L1 of FdPV2. The principle of such an ELISA is the expression of the antigen as GST-fusion protein. The viral antigen in the form of this fusion protein could be bound to the ELISA plates coated with glutathione linked casein. The sera are tested to contain antibodies against the antigen, which is attached to the plates over the affinity of GST to glutathione. We already successfully used this technique to determine the seroprevalence of CPV1 and CPV3[26] and EcPV2[27] in corresponding populations. As a negative control in our study we used the major capsid protein L1 of CPV1.

The aim of the present study was to shed light on the prevalence of FdPV2 in cats that do not suffer from any dermatological conditions. First, we determined the DNA prevalence in skin samples using specific primers and compared it to the prevalence found in BISC. According to the literature, FdPV2 was given a straight forward role of infecting feline skin cells and inducing BISC.[28] If this was true, samples from normal skin should contain significantly less virus DNA. However, considering that other PV cause frequent subclinical infections, the working hypothesis of our study was that there should be no significant difference between healthy cats and cats with BISC in genoprevalence of FdPV2. Second, we wanted to determine the seroprevalence of FdPV2 and compare it with its genoprevalence. This would give us a first comprehension on how many cats that carried the virus on their skin reacted with seroconversion.

Materials and methods

Sampling from cats

For this study, we sampled 100 cats that had been presented to the Clinic of Small Animals, Vetsuisse-Faculty, Zurich, Switzerland. In order to screen “healthy” cats, we included only cats that suffered from issues without any known relationship to PV infection, i.e. any kind of skin lesions.

The included cats showed a wide distribution in their age range. The youngest cat was only 3 months old whereas the oldest one was 17 years old. The age of 10 cats was unknown. The median age of the cats was 7 years. Twenty-two cats were less than one year old. From the 100 cats, 60 were male (44 neutered) and 40 were female (20 neutered). 76 cats were mixed breeds and 24 cats were purebred cats or descendants of two different purebred cats, respectively (Table 1).

Six owners allowed us to sample multiple cats. Five owners had two cats each that shared the same household. One owner even provided five cats that live on the same farm. As the cats were all presented for castration, they were healthy and young. The age varied from 0.4 to 1.1 years (median = 0.7). However, the age from the five cats that live on the farm was not exactly known.

Skin cell samples were taken with a Cytobrush cell sampler (Deltalab; Barcelona, Spain). Two samples were taken from each cat. The first sample was taken from the haired skin around the mouth in the area where the left vibrissae are located. The second sample was taken from the right front paw, interdigitally between P3 and P4. If the described areas were not accessible for any reason (e.g. injury or bandage), the corresponding areas on the contralateral side were used for sampling. Briefly, a Cytobrush was wetted in 0.9% sterile NaCl solution and rubbed with rotating movement for 30 seconds on the skin of the described area. The handle of the Cytobrush was then cut off and the brush part placed in a sterile 1.5ml Eppendorf tube.

Serum samples were taken during routine diagnostics not related to our study or when a new venous catheter was placed. To define a reliable seroprevalence, the ability to produce antibodies must be given. Therefore, animals with a known or suspected history of immunodeficiency or under treatment with immunosuppressive drugs were not included. If a current blood count of a candidate was available, it was checked and cats with hints for immunodeficiency were excluded.

Two cats with lesions that had been histologically confirmed as BISC served as positive controls. The Cytobrush samples were taken directly from the BISC lesions. One cat was sampled at two lesions on the neck whereas the other cat was sampled at one lesion on the forehead resulting in a total of 3 samples. Serum samples were taken during routine diagnostics.

As a negative control, Cytobrush and serum samples were taken from 5 specific pathogen-free (SPF) cats.[29] The Cytobrush samples were taken from the same locations as described above. All serum and Cytobrush samples were stored at -20°C until further analysis.

Breed	Number of Cats
Mixed breed	76
Persian	4
British Shorthair	3
Bengal	2
Burmese	2
Maine Coon	2
Siamese	2
Siberian	2
Birman	1
Egyptian Mau	1
Norwegian forest cat	1
Norwegian forest cat x Persian	1
Ocicat	1
Ragdoll	1
Turkish Van	1

Table 1. Breed distribution in the sample cat population (n=100).

PCR

DNA was extracted from the Cytobrush samples using QIAamp® DNA Mini Kit (Qiagen; Basel, Switzerland) according to the manufacturer's protocol with some modifications. The amounts of buffer ATL, proteinase K, buffer AL and ethanol were doubled to ensure that the complete Cytobrush was covered. The extracted DNA was finally dissolved in 100µl of buffer AE.

Several published primer pairs were evaluated for their sensitivity to amplify PV DNA from feline skin samples.[15,18,30–35] Additionally, new primers were designed to amplify parts of the FdPV2 E1, E6 and L1 ORF, respectively (table 2). The selected primer sets were ordered (Microsynth; Balgach, Switzerland) and tested for their sensitivities. PCR using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich; St. Louis MO, USA) was performed. As template DNA a dilution series of the plasmid containing the FdPV2 genome (see chapter "antigen production") reaching from 10^9 to 10^{-1} copies/µl was used. Amplification conditions were adopted from the literature. For the self-designed primers, the amplification conditions described above were used. The products were analysed by electrophoresis in a 1% agarose gel containing 0.01% (w/v) ethidium bromide. The most sensitive set of primers was chosen for further analysis.

Quantitative real-time PCR (qPCR) was performed using the iCycler iQ™ Real-Time PCR Detection System with the corresponding transparent 96-well plates (Bio-Rad; Hercules CA, USA). Reactions contained 10µl iQ™ SYBR® Green Supermix (Bio-Rad; Hercules CA, USA), 0.6µl forward primer (10µM), 0.6µl reverse primer (10µM), 3.8µl sterile water and 5µl template DNA. Negative controls contained no template DNA but additional 5µl of sterile water. Plates were sealed with iCycler iQ™Optical Tape (Bio-Rad; Hercules CA, USA) and centrifuged at 2000rpm for 10 minutes before performing qPCR. The following amplification conditions were used: 3 minutes at 95°C, 41 cycles of 10 seconds at 95°C and 30 seconds at 60°C and 1 cycle of 1 minute at 95°C and 1 minute at 55°C. Afterwards temperature was raised by 0.5°C per cycle during 84 cycles of 10 seconds to create the melt curve.

As a reference gene, feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen. A set of new primers was designed to amplify cat's GAPDH. The nucleotide sequences of the resulting set of primers (fGAPDH_qPCR) can be found in table 2. qPCR was performed using the same protocol as described above.
Calibration curves were created with dilution series of plasmids containing the desired DNA. For FdPV2, the plasmid containing the entire FdPV2 DNA (see chapter "antigen production") was used. For GAPDH, PCR was performed to amplify the desired genome sequence using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich; St. Louis MO, USA) and the primers catGAPDH_f (5'-TCA TCA TCT CTG CCC CTT CT-3') and catGAPDH_r (5'-GTG AGC TTC CCA TTC AGC TC-3'). Amplification conditions were the same as described above (see chapter "Antigen production"). The obtained product was cloned using the TOPO TA Cloning® Kit with the pCR®2.1-TOPO® vector and chemically competent E.coli strain TOP10 cells (Invitrogen; Carlsbad CA, USA). The plasmids were extracted using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich; St. Louis MO, USA) and verified by sequencing (Microsynth; Balgach, Switzerland).

Antigen production for ELISA

The FdPV2 L1 coding sequence (CDS) was amplified by PCR using Phusion™ High-Fidelity DNA Polymerase (Finnzymes; Espoo, Finland). A pBluescript II KS(+) vector (Agilent Technologies; Santa Clara CA, USA) containing the whole genome of FdPV2 served as template.[15] Primers were specifically designed to amplify the FdPV2 L1 CDS lacking the first ten (5') codons. For the purpose of cloning, *Bam*HI sites were added at the 5' ends of the primers. The nucleotide sequences of the resulting set of primers (FdPV_L1_long) can be found in table 2. Amplification conditions were: 5 minutes at 95°C, followed by 40 cycles of 1 minute at 95°C, 1 minute at 58°C, and 1.5 minutes at 72°C with a final elongation step of 5 minutes at 72°C. The amplimer was cloned into the *Bam*HI site of the pGEX-6P-1 vector (Pharmacia Biotech; Uppsala, Sweden) under the control of an isopropyl-β-D-thio-galactoside (IPTG) inducible T7 promoter. The resulting plasmid pGEX FdPV2 L1 was then propagated in *E.coli* strain DH10B.

Primer set		Nucleotide sequence
FdPV_L1_long	Forward	5'-CGA CGG ATC CTT ATA TCT CCC ACC CTC CCC TG-3'
	Reverse	5'-AAT AGG ATC CTC ATT TGC GGG TGC GTT-3'
FdPV2_E1	Forward	5'-CAG CTC CCA GTC TCC TAA CG-3'
	Reverse	5'-GCT GTG CCA TTA TCT GAG CA-3'
FdPV2_E6	Forward	5'-GCG TAT TTT GCG GAA CAC TT-3'
	Reverse	5'-CCA GAT CCC TGT GCA AAA AT-3'
FdPV2_L1	Forward	5'-CCC GAA ACA GAC GCA ATT AT-3'
	Reverse	5'-TAT TGC CAA ACC CAG TGT CA-3'
fGAPDH_qPCR	Forward	5'-GTG GAG GGA CTC ATG ACC AC-3'
	Reverse	5'-GTG AGC TTC CCA TTC AGC TC-3

Table 2. Nucleotide sequences of novel primer sets used in this study. *Bam*HI site within the FdPV_L1_long are underlined.

To control the correct insertion of the FdPV2 L1 sequence, the extracted plasmids were analysed by restriction enzyme digests and the integrity of the sequence was confirmed by sequencing (Microsynth; Balgach, Switzerland). The isolated plasmid DNA was transformed into *E.coli* strain BL21(DE3), which express the T7 polymerase upon IPTG induction. Protein expression was performed as described previously with minor modifications.[36] The bacterial cells were grown in 15ml LB medium containing 100µg/ml Ampicillin at 25°C with shaking (220rpm) until an OD_{600} of 0.3. Protein expression was induced by adding 0.25mM IPTG and the cells were incubated over night at 25°C with shaking (220rpm). After centrifugation the pelleted bacteria were solved in 1.5ml buffer L (40mM Tris pH 8.0, 200mM NaCl, 1mM EDTA and 2mM DTT) supplemented with Complete Protease Inhibitor Cocktail (Roche; Mannheim, Germany) and lysed by sonication. ATP (2mM) and MgCl (5mM) were added and the lysate was incubated for 1 hour at room temperature. Urea was slowly added over 5 minutes to a final concentration of 3.5M. After another incubation of 2 hours at room temperature, the mixture was split into 2 equal parts and dialysed against buffer L using 7K MWCO Slide-A-Lyzer® Dialysis Cassettes (Thermo Scientific; Rockford IL, USA) over night at 4°C. Buffer was exchanged and dialysis was carried on for 2 hours. After centrifugation the obtained antigen mix was diluted 1:1 with glycerol 100% and stored at -20°C.
The protein expression procedure was simultaneously performed with three different *E.coli* strain BL21(DE3) cultures containing different pGEX-6P-1 vector derivatives. The first contained the FdPV2 L1 CDS fused to the GST CDS whereas the second contained a CPV1 L1 CDS fused to the GST CDS.[26] The third culture contained the GST CDS only. All ELISA assays reported in this study were performed with antigen from the same lot of antigen production.

GST capture ELISA

Polysorb 96-well plastic plates (Nunc; Roskilde, Denmark) were coated at 4°C over night with 50mM sodium carbonate buffer pH 9.6 containing 0.2% glutathione casein (kindly provided by Martin Müller DKFZ, Heidelberg, Germany). The plates were then washed three times with PBS buffer containing 0.3% Tween 20 (PBS-T) and blocked at 37°C for 1h with casein buffer (PBS-T containing 0.2% casein). After washing three times with PBS-T, the plates were incubated at 37°C for 1h with the GST tagged antigen diluted 1:10 in casein buffer. The plates were washed again three times with PBS-T.
The sera had been diluted 1:500 in casein buffer, mixed with an equivalent of lysed untransformed *E.coli* strain BL21 (DE3) and incubated at 4°C for 30min to block reactions with contaminating bacterial proteins.[25,26,36] The plates were then incubated with the prepared sera at 37°C for 1h and washed again three times with PBS-T. Goat Anti-Feline IgG conjugated to Horseradish Peroxidase (HRP) (Southern Biotech; Birmingham AL, USA) diluted 1:1000 in casein buffer was added as secondary antibody and the plates were incubated again at 37°C for 1h. After washing six times with PBS-T, substrate (78mM CH_3COOH, 24mM CH_3COONa, 50mM NaH_2PO_4, 2mM ABTS [Roche; Rotkreuz, Switzerland] with 1.25mM H_2O_2

applied shortly before use) was added. Absorbance was measured after 45min at 405nm in a Sunrise™ microplate reader (Tecan; Männedorf, Switzerland).
The cat sera were tested in triplicates against the antigen FdPV2 L1-GST and, as a negative control, against CPV1 L1-GST. For a subset of samples the ELISA was repeated. The according samples were then tested in duplicates against CPV1 L1 and against GST alone. In order to normalize the results of the different plates, the same positive and negative control sera were used on every plate. No serum was added in six wells serving as a plate control.

Data analysis and presentation

The C_q-values obtained from qPCR were converted into absolute numbers of copies of FdPV2 and GAPDH in each sample using the equation of the corresponding calibration curve. In order to obtain comparable results, in each sample the absolute number of FdPV2 copies was divided by the corresponding absolute number of GAPDH copies.
Serum samples were tested in triplicates in ELISA. To prevent outlier results from influencing the data, the median of the three observed values was used for further analysis. Plate to plate variability was compensated by dividing every value by the mean of the control sera values from the corresponding plate and multiplying the result by the mean of all control sera from all plates.
Different methods of setting a cut-off value (COV) were evaluated for the qPCR and ELISA. In method 1 (named SNC), the COV was set equal to the strongest reaction of a negative control serum. In method 2 (named WPC), the weakest reaction of a positive control serum was used as reference. In method 3 (named MSWC), the mean of the values used in methods 1 and 2 was taken.[26] In method 4 (named XBP2S), the X-Bar-plus-2s procedure was applied.[25,37,38] The mean of all negative control samples was calculated and 2 standard deviations were added.
Data was organised using Microsoft Excel 2010 (Microsoft; Redmond WA, USA) and further statistical analysis was done by IBM® SPSS® Statistics version 20 software (IBM; Armonk NY, USA). Figures were generated using R (Free Software Foundation; Boston, USA). Boxplots are produced using R with default settings. The solid bar represents the median, the box range from the first to the third quartiles, and the whiskers extend to the lowest and highest datum still within 1.5 times the interquartile range. Data not included within the whiskers are individually presented.

Results

PCR

In order to test the skin samples for the presence of FdPV2 specific DNA, a qPCR was developed and applied. First, different FdPV2 specific primer sets were compared and the most sensitive was chosen. Second, the amount of FdPV2 DNA was determined using qPCR with GAPDH as reference gene. Third, a COV was set. Fourth, the DNA-prevalence of the sample population was determined.

Evaluation of PCR primer pairs for the detection of FdPV2 specific DNA

The sensitivities of different primer sets were tested with a dilution series of a plasmid containing the FdPV2 DNA. We tested ten of the published primer sets that had been used to screen samples for FdPV2. Using the same amplification conditions as described in the according publications, six of these ten primer sets were not able to detect the plasmid even in a concentration of 10^9 copies/µl (Table 3).

Primer set	Minimal amount (copies/µl)
$E5^+/E5^-$ [30]	$>10^9$
IFNR-2/IDNT-2 [6,30,38]	$>10^9$
jmpSA-F/jmpSA-R [31]	$>10^9$
MY09/MY11 [3,7,21,24,25,31,32,38,39]	$>10^9$
NO1/NO2 [30]	$>10^9$
PV3/PV5 [33]	$>10^9$
FAP59/FAP64 [3,5–7,18–21,23,34,38,39]	10^8
CP4/CP5 [14,35]	10^6
FdPV2_L1	10^6
A16/A37 [15]	10^4
FdPV2_E6	10^2
JMPF/JMPR [3,7,18–21,23–25]	10^2
FdPV2_E1	10^0

Table 3. Minimal amount of FdPV2 plasmid DNA (copies/µl) needed for a positive PCR result. $>10^9$ = no positive result within the range of the used DNA dilution series (10^9-10^{-1} copies/µl).

For the other four primer sets, the minimal amount of DNA in the sample needed for detection varied from 10^2 - 10^8 copies/µl. All of the three newly designed primer sets succeeded to detect the template DNA. The minimal amount needed varied from 1 - 10^6 copies/µl. The primer set designed to amplify the FdPV2 E1 ORF turned out to be the most sensitive of all tested sets. FdPV2 plasmid DNA in a minimal amount of one copy/µl was enough to repeatedly obtain positive PCR results. We decided therefore to use this primer set for our qPCR. The results of the conventional PCR are listed in table 6 (see appendix).

Calibration curves for calculation of copy numbers of FdPV2- and GAPDH-DNA

Serial dilutions of cloned FdPV2 and GAPDH DNA templates were used to evaluate the equations to convert the C_q values into copy numbers of DNA molecules. The equations of the calibration curves were for GAPDH C_q = -3.98x log(molecules) + 39.34 (R^2 = 0.9767) and for FdPV2 C_q = -3.99x log(molecules) + 40.16 (R^2 = 0.9951)

(Figure 1). This resulted in efficiencies of 78.3% for the GAPDH-qPCR and 78.1% for the FdPV2-qPCR.

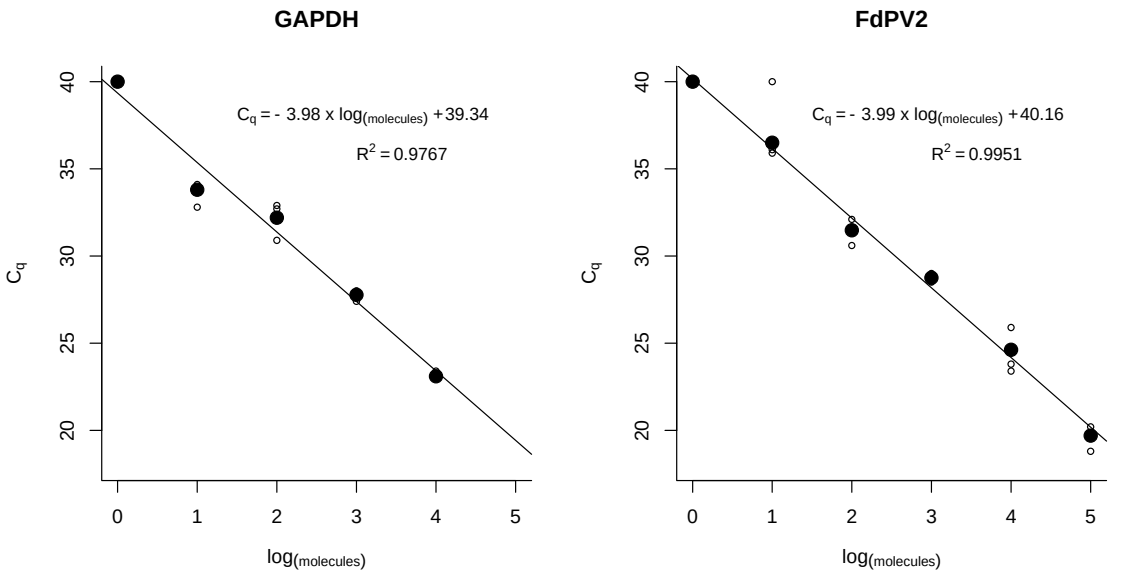

Figure 1. Calibration curves for qPCR. Dilution series of cloned feline GAPDH cDNA and FdPV2 DNA were used as template and qPCR was performed using specific primer sets (see above). The resulting equations of the calibration curves were for GAPDH C_q = -3.98x $\log_{(molecules)}$ + 39.34 (R^2 = 0.9767) and for FdPV2 C_q = -3.99x $\log_{(molecules)}$ + 40.16 (R^2 = 0.9951). These equations were used to quantify the results of the qPCR using the same primer sets but the DNA extracted from the Cytobrush samples as templates.

Measurement of the FdPV2 DNA copies of the Cytobrush samples

The readily established qPCR assay for FdPV2 was used to evaluate the viral DNA in the Cytobrush samples collected from the healthy sample population. The results were quantified using calibration curves and converted to comparable values using feline GAPDH as a reference gene. The log-transformed ratios are shown as box plots in figure 2A. The medians of the negative controls were significantly lower than these from the positive controls. The medians of the samples were for both, the samples from the head as well as the samples form the paw between the positive and the negative controls. To further explore the distribution of the ratios, the log-transformed ratios were plotted as histogram and shown in figure 2B. The distribution of the transformed ratios of the samples collected from the heads as well as these collected from the paws resembled a normal distribution with additional data points at the lower end. To better visualize the distribution of the data, the normal distribution with the mean and the standard deviation of the positive and negative controls were included. The sum of the two normal distribution curves approximated the curve of the density function from the sample population.

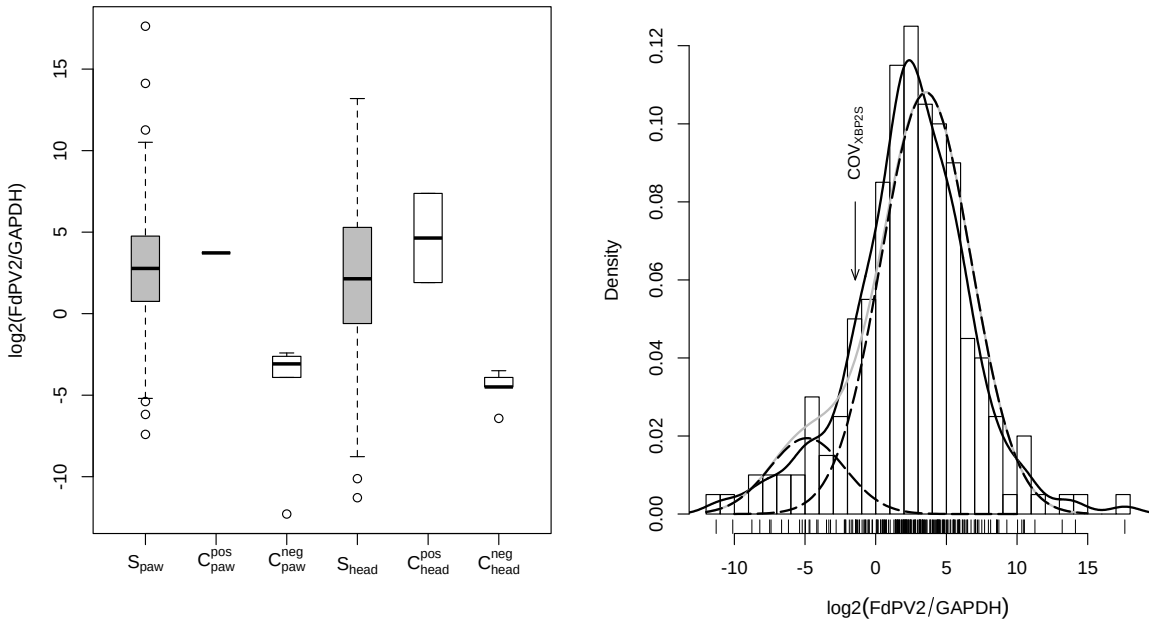

Figure 2. (A) Boxplot of the log-transformed ratios of FdPV2 to GAPDH molecule numbers. The positive controls (C^{pos}_{paw}, C^{pos}_{head}) and the negative controls (C^{neg}_{paw}, C^{neg}_{head}) are shown as white boxes and the samples (S_{paw}, S_{head}) as grey boxes. The boxes and whiskers are used as defined in the method section. **(B)** Histogram of log-transformed ratios of FdPV2 to GAPDH molecule copy numbers. A density plot is overlaid as solid black line and the normal distribution with mean and standard deviation of the negative and positive control samples are shown as dashed lines. The sum of both normal distributions is shown as a solid grey line.

Assessment of a meaningful cut-off value (COV) for the qPCR

In order to group the samples into positive and negative results, a COV had to be set. Different methods of setting a COV were compared (Table 4). The highest prevalence resulted if either the SNC method (COV = 0.188) or the XBP2S method (COV = 0.315) was applied. With either method 169 out of 189 samples would have been classified as positive, which corresponded to a prevalence of 89%. Using the MSWC method (COV = 1.970), 129 samples could be counted as positive (prevalence = 68%). The lowest value for the prevalence resulted from an application of the WPC method (COV = 3.754). Only 111 samples were positive in that case, which corresponded to a prevalence of 59%. For further analysis of the data, we used the COV described in XBP2S as this is a widely accepted method for setting a COV.[37]

COV	p+	p-	h+	h-	p+/h+	p-/h-	p+/h-	p-/h+
SNC	93	7	87	13	82	2	11	5
WPC	67	33	52	48	40	21	27	12
MSWC	74	26	64	36	51	13	23	13
XBP2S	90	10	83	17	77	4	13	6

Table 4. Number of positive and negative cats when applying different COV. (criterions and combinations of are as followed. p+: paw sample positive; h+: head sample positive; p-: paw sample negative; h-: head sample negative)

Determination of the genoprevalence of FdPV2 in the sample population

The determined COV was used on the ratios of the sample population. Out of the 200 DNA samples, 189 could be used for the study. In nine samples, the qPCR with FdPV2 E1 specific primers amplified an unspecific by-product as recognized by analysis of the melting curves. The resulting quantification cycle (C_q) value was false high. Eight of these nine false high values were above the COV after quantification and therefore taken out of further analysis. One value was below the COV and treated as a normal negative result. In three other samples amplification of GAPDH failed. Calculating the copy number of FdPV2 per GAPDH was consequently not possible and the samples were taken out of further analysis. The determined C_q values varied from 14.7 to 37.4. Negative samples were labelled “n/a”. In order to calculate a comparable value, C_q was set as 40.

Among the 189 samples used for further analysis, the calculated FdPV2 DNA copies per GAPDH varied from $4.0 \cdot 10^{-4}$ to $9.3 \cdot 10^{4}$ in the samples from the head and from $5.9 \cdot 10^{-3}$ to $2.0 \cdot 10^{5}$ in the samples from the paw. Among the negative control samples FdPV2 DNA copies per GAPDH varied from $1.2 \cdot 10^{-2}$ to $8.8 \cdot 10^{-2}$ in the samples from the head and from $2.0 \cdot 10^{-4}$ to $1.8 \cdot 10^{-2}$ in the samples from the paw. In nine of these ten samples no specific FdPV2 DNA could be amplified in qPCR. C_q was therefore set as 40 as described above. However, in one paw sample specific FdPV2 DNA could be amplified. C_q was 35.9. The calculated number of FdPV2 DNA copies per GAPDH was $1.6 \cdot 10^{-1}$.

No relationship between the copy numbers in head and paw samples could be found. A high number of copies in the head sample was not necessarily accompanied by a high number of copies in the corresponding paw sample. The positive control samples, which had been taken directly from BISC lesions, varied from only $3.7 \cdot 10^{0}$ to $1.7 \cdot 10^{2}$.

A COV was set at 0.367 according to method XBP2S as described above. 169 samples were above the COV whereas 20 samples remained below. From these 20 samples, 13 were taken from the head and seven from the paw. Ninety-eight cats had at least one positive sample and were therefore counted as FdPV2 DNA positive. The two FdPV2 DNA negative cats both had two samples of sufficient quality (see exclusion criteria above) and did not have any relation to each other. Summarized, the DNA prevalence of FdPV2 in the studied population was determined to be 98%. Further statistical analysis was not performed as the group of negative samples is too small to expect meaningful results.

GST capture ELISA

In order to test the serum samples for the presence of FdPV2 specific antibodies, an ELISA was developed and applied. First, the antigen coating of the plates and the measurement of antibodies in the control sera were tested (see tables 5 and 6 in appendix). Second, the antibody titres of all samples were determined and normalized (see table 8 in appendix). Third, a meaningful COV was set. Fourth, the seroprevalence of the sample population was determined.

Assessment of the COV for the ELISA

We evaluated the four methods used already for the qPCR to calculate the COV for the ELISA readouts.

The corresponding seroprevalences for FdPV2 varied considerably. Setting the COV equal to the strongest reaction of any negative control (SNC, COV = 0.292) resulted in 27 positive samples. If set equal to the weakest reaction of any positive control (WPC, COV = 1.743), no sample serum could be considered as positive. Taking the mean of these two values (MSWC, COV = 1.017) gave only one positive sample. The COV of the X-Bar-plus-2s method (XBP2S, COV = 0.315) was close to the one obtained by the SNC method. If applied, 24 samples were positive. For further analysis, the COV was set relying on the XBP2S method as we used this method in the qPCR already.

COV	F+	F-	F+/C+	F-/C-	F+/C-	F-/C+
SNC	27	73	2	73	25	0
WPC	0	100	0	98	0	2
MSWC	1	99	1	98	0	1
XBP2S	24	76	2	76	22	0

Table 5. Number of positive and negative serum samples when applying different COV. (F+/-: FdPV2 ELISA positive/negative; C+/C-: CPV1 ELISA positive/negative)

The reaction of the sera against CPV1 antigen was tested as a negative control in a separate ELISA. A GST-CPV1 L1 fusion protein instead of a GST-FdPV2 L1 was used to coat these assay plates. All other reagents and dilutions were applied as in the FdPV2 specific assay. Similar to the FdPV2 ELISA, an individual COV was assessed with the XBP2S method and set to 0.472 for the CPV1 ELISA.

Analysis of antibody titres in cat serum by ELISA

The serum samples were screened for antibodies against FdPV2 and, as a negative control, against CPV1 using a GST capture ELISA. Absorbance was measured after 45min at 405nm. The sample sera reacted against FdPV2 with an OD ranging from 0.154 to 1.094 (mean = 0.301) and against CPV1 with an OD ranging from 0.162 to 1.096 (mean = 0.242). Setting the COV using the XBP2S method resulted in a COV of 0.315 for FdPV2 as described above and 0.472 for CPV1. The reactions against FdPV2 of 24 serum samples were above the COV and could thus be considered positive while 76 serum samples were counted as negative. Two serum samples showed a reaction against CPV1 above the according COV (Figure 3).

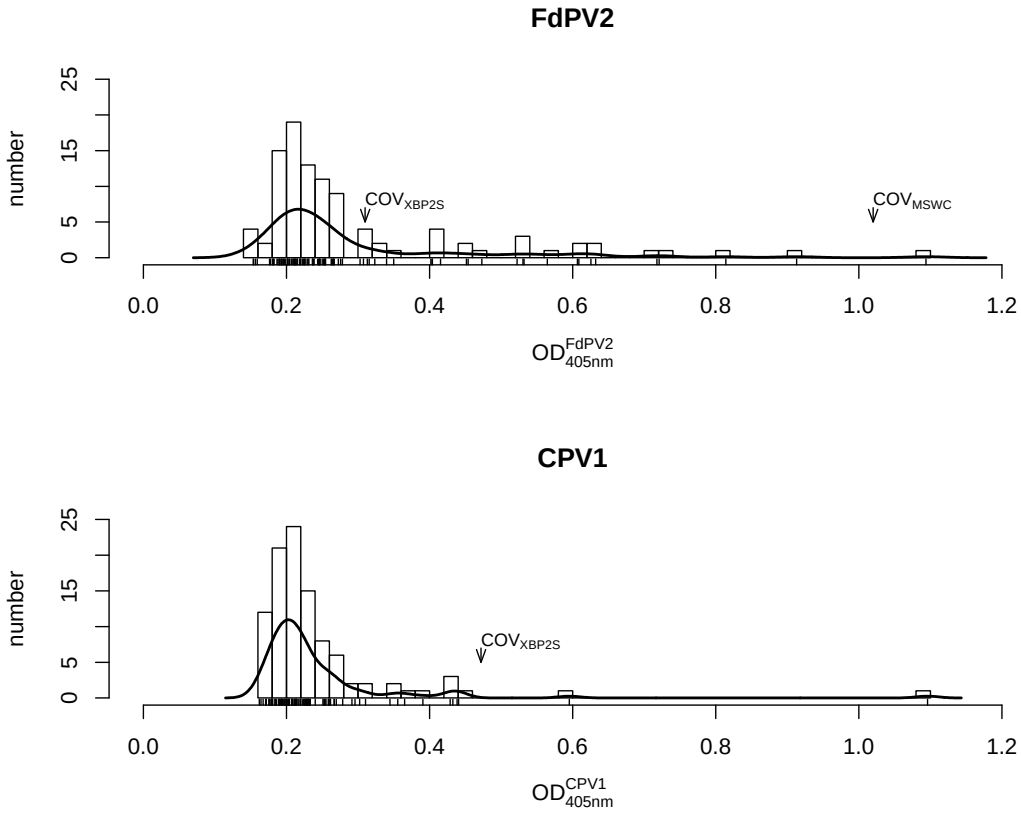

Figure 3. Histogram plot of the corrected ELISA OD values from the cat's serum samples (without control sera) for the FdPV2 specific antibodies (**upper panel**) and CPV1 specific antibodies (**lower panel**), respectively. Ranges of the bins are 0.02 OD. Individual values are indicated as rug plot below the bars and the density of the distribution is shown as solid line. Setting the COV for the FdPV2 specific ELISA as described in the XBP2S method (COV = 0.315) resulted in 24 positive samples. Using the MSWC method (COV = 1.017) resulted in only one positive sample (see arrows). Setting the COV for the CPV1 specific ELISA as described in the XBP2S method (COV = 0.472) resulted in 2 positive samples.

To incorporate the CPV1 control in the data analysis, the corrected OD values of the FdPV2 specific ELISA were plotted against the corrected OD values of the CPV1 specific ELISA (Figure 4). As mentioned above, two of the 24 positive serum samples showed a reaction against CPV1 with an OD above the according COV (circles on the right side of the vertical and above or close to the diagonal line on figure 4). The ELISA was repeated with these samples. CPV1 L1 (tagged to GST) and GST alone were used as antigens. In both samples the reactions against GST alone were as strong as the reactions against CPV1 L1-GST. The samples were therefore categorised as negative for antibodies against CPV1 as well as against FdPV2. The seroprevalence of FdPV2 was corrected down to 22%.

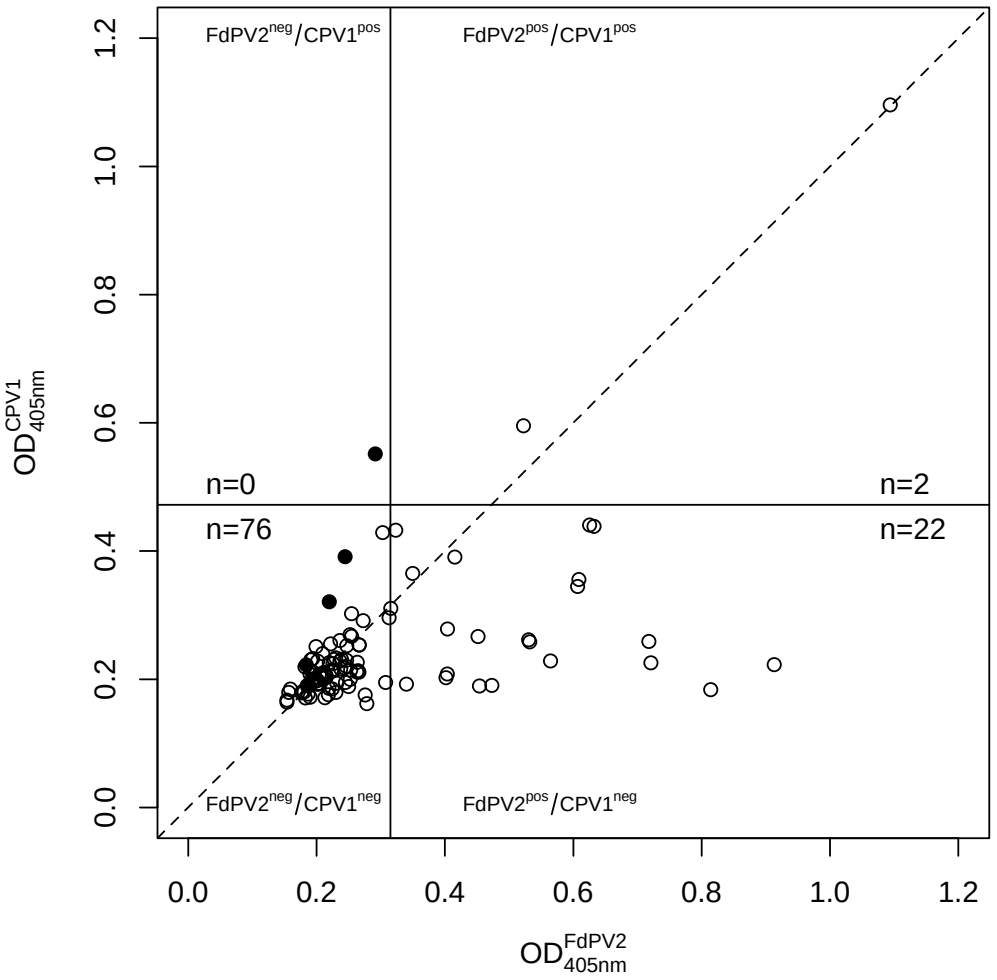

Figure 4. OD values of the FdPV2 specific ELISA versus OD values of the CPV1 specific ELISA. The respective COVs of the assays as determined by the XBP2S method is shown as vertical line for the FdPV2 ELISA and as horizontal line for the CPV1 ELISA. To visualize data points with values of the CPV1 OD above the FdPV2 OD, a diagonal line is drawn. Samples are shown as circles and negative controls as black dots. The positive control is out of the range of the axis.

The OD of the negative control sera ranged from 0.184 to 0.291 (mean = 0.225) and the one of the positive control sera from 1.743 to 2.041 (mean = 1.892) in the FdPV2 specific ELISA (see tables 7 and 8 in appendix). Bonferroni statistical test was used to compare the mean OD of the sample sera with those of the positive and negative control sera, respectively. The mean OD of the positive control sera was significantly higher than the mean OD of the sample sera (p=0.000), whereas the mean OD of the negative control sera did not differ significantly from the mean OD of the sample sera (p=0.986). Fisher's Exact Test was used to compare subgroups of the sample population (see figure 6 in appendix). No difference in seropositivity could be found between purebred and mixed breed cats (p=0.386). The sera of male cats were as often tested positive as the sera of female cats (p=0.448). There was no difference between intact individuals and castrated ones (p=0.061). The seropositive cats had a median age of 12.0 years. This is significantly older (Univariate Analysis of Variance, p=0.000) than the negative cats that had a mean age of 4.3 years. Yet, the age of

three positive and seven negative cats was not known. The 15 cats living in multiple cat households were all tested negative.

Correlation of ELISA and PCR

Results obtained from ELISA and PCR were compared. The log-transformed ratios from the Cytobrush samples isolated from head were plotted against the ones from the paw and the size of the dot corresponded to the OD value of the FdPV2 specific ELISA (Figure 5).

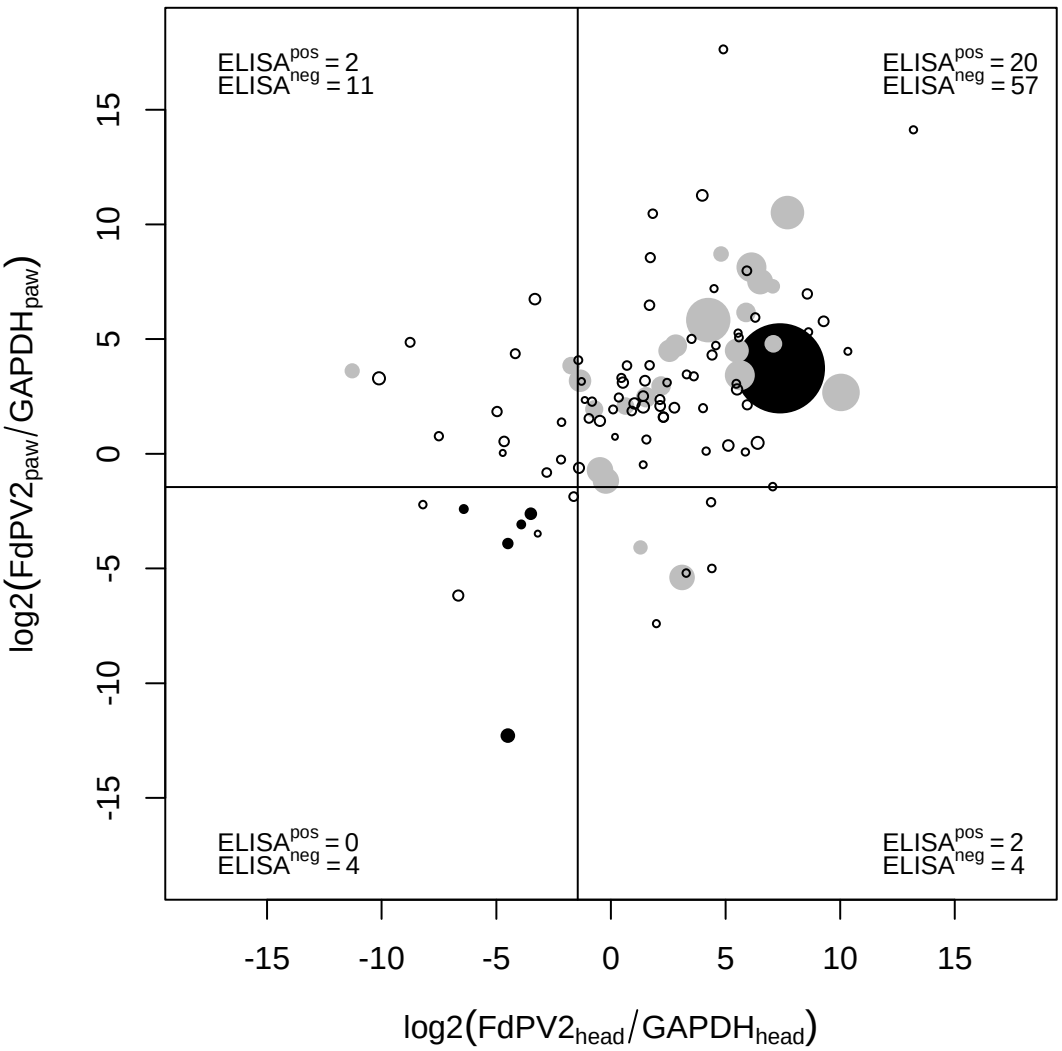

Figure 5. Scatter plot of log-transformed qPCR copy numbers ratios of FdPV2 DNA to GAPDH DNA in head and paw samples and the according ELISA OD value from the serum sample. White circles represent FdPV2 ELISA negative cats from sample population, grey dots represent FdPV2 ELISA positive cats, and black dots represent positive and negative control cats. The drawing sizes of the circles/dots indicate the ELISA OD value. The vertical and horizontal lines are set at the level of the COV (0.202). The numbers of FdPV2 ELISA positive and negative cats in each of the four categories describing the genoprevalence of FdPV2 on head and paw are specified.

Data points corresponding to cats with similar ratios of FdPV2 DNA to GAPDH DNA on paw and head would lie close to a diagonal line through the origin with a slope of 1. This prospective correlation was not obvious. However there was a tendency of the larger circles (corresponding to FdPV2 ELISA positive cats) within the region of qPCR positive in paw- and head-samples and small circles within the region of negative qPCR samples. Though, some cats had different ratios of FdPV2 and GAPDH DNA in the paw- and head-samples.

Cats tested positive in ELISA showed a large variation in PCR results. The lowest observed copy number of FdPV2 per GAPDH was only $4.0 \cdot 10^{-4}$ whereas the highest value was $1.4 \cdot 10^{3}$. Two seropositive cats had less than one copy of FdPV2 per GAPDH in both samples and two other had one negative PCR sample each. None of the positive control samples had more than 200 copies of FdPV2 per GAPDH. The lowest FdPV2 DNA content in a sample from the seronegative cats was $9.0 \cdot 10^{-4}$, while the highest was $2.0 \cdot 10^{5}$. One seronegative cat had more than $9.0 \cdot 10^{3}$ copies of FdPV2 per GAPDH in both samples. The two cats that remained DNA negative were tested negative in ELISA as well. Overall the seronegative cats had on average almost 15 times as many copies of FdPV2 DNA (mean $1.4 \cdot 10^{3}$) than seropositive cats (mean $1.0 \cdot 10^{2}$).

Discussion

In this study, we determined the prevalence of FdPV2 in a population of dermatologically unremarkable cats by two methods, (1) detection of viral DNA, (2) detection of specific serum antibodies. We could amplify specific FdPV2 DNA from 98% of the skin samples. This DNA prevalence was thus much higher than the one previously reported of 52%.[19] It was comparable to the FdPV2 DNA prevalence found in BISC and viral plaques. With this data we could confirm our hypothesis that there would be no significant difference in DNA prevalence in samples from BISC lesions or from normal skin.

Various other studies reported the screening of feline skin samples for PV by immunohistochemistry (IHC). In those studies, IHC was used to detect the major capsid protein, which is the product of the L1 ORF. This ORF is only expressed in productively PV infected cells late in the viral replication cycle.[1,21] Unproductively infected cells or cells with virus in the very early stage of the replication cycle cannot be detected by immunohistology.

As PCR detects genome sequences regardless of gene expression, it is suggested to be the more sensitive method for PV detection than IHC.[6] Furthermore, PCR is easier to apply than IHC. Consequently, in most of the other studies PCR was used for PV detection. An overview of the studies that used PCR for PV detection in skin samples of cats is given in table 6 (see appendix). In some of the studies, specific DNA of FdPV2 could be detected using broad range or specific primers. The reported FdPV2 DNA prevalence rates of these studies, however, are often much lower than the DNA prevalence found in our study. In the majority of the existing studies, DNA was extracted from formalin-fixed biopsy samples. Formalin-fixation is known to cross-link and fragment genomic as well as viral DNA.[39] A low yield of intact DNA might result, which lowers the sensitivity of PCR either by decreased primer affinity or by strand-breaks within the targeted sequence. Likewise, Munday et al. showed that amplification of PV DNA from cotton-tipped swabs was a more sensitive method than amplification from formalin-fixed tissue.[7] In our study, we used Cytobrushes to take skin cell samples. Cytobrushes are normally used to take cell samples from mucosae. Chalvardjian et al. compared the uptake of cells of cotton-tipped swabs and Cytobrushes by performing endocervical sampling in women. The samples taken with a Cytobrush contained on average at least 17 times more endocervical cells than the samples taken with a cotton-tipped swab in 87% of the cases.[40] In our study, we took the Cytobrush samples from the haired skin. Only 3 out of 200 Cytobrush samples were negative in qPCR for GAPDH and, therefore, 197 samples were apparently of sufficient quality. This demonstrates that Cytobrushes are a very useful and efficient tool for collecting cell samples even from the haired skin.

Comparing different types of feline skin lesions, the DNA prevalence of FdPV2 seemed to vary considerably. Low rates of FdPV2 were found especially in ISCC and non-SCC lesions. However, it is difficult to compare all the published studies among each other and with our own study, since many different primer sets were used for PCR. It was shown that consensus primers are not sensitive enough to

detect small amounts of specific FdPV2 DNA.[19] In order to choose the most sensitive primer set, we evaluated 10 of the published and 3 novel primer sets. Every primer set was tested with the same template DNA, a dilution series of a plasmid containing the entire FdPV2 genome and the recommended cycling protocol in a conventional PCR. The results revealed a remarkable difference in the sensitivity among them. In our evaluation, six primer sets that had been used in other studies, failed to detect FdPV2 DNA even at a predetermined concentration of 10^9 copies/µl. This might be partially explained by the fact that we used different cycling devices and polymerase suppliers. However, low sensitivity of the used primer sets may still be a major reason why other studies detected only low rates of FdPV2 in certain types of lesions. A causative role of FdPV2 should not be suggested only by a higher detection rate in certain types of lesions if different PCR with different primer sets and amplification protocols were used. One study found FdPV2 DNA in 34 of 88 (39%) skin samples from normal skin.[19] However, we could detect FdPV2 DNA in 169 of 189 (89%) skin samples. The much higher rate could be explained again with the sensitivity of the primers as the primer set used in our study turned out to be 100 times more sensitive, detecting FdPV2 DNA even at a template concentration as low as one copy per microliter.

The primers used in this study were designed to amplify the genome sequence of the FdPV2 E1 ORF. As in other PV, the 1805bp FdPV2 E1 ORF is the largest ORF in the genome.[1,15] Regarding the PV life cycle, the E1 ORF product is required for initiation and elongation of DNA synthesis. Together with the L1 and the L2 ORF, it is more conserved than the other ORFs.[1] One study even identified two 21bp regions in the E1 ORF as being the most highly conserved regions among all PV species examined.[33] Of note, the specificity of this primer set could still be confirmed by sequencing the amplified products.

GAPDH was chosen as reference gene as it is generally accepted and recommended in the literature.[41] Calculating the number of FdPV2 copies per GAPDH allows comparing samples with different amounts of cells and total DNA. Nevertheless, remarkable differences could be found from cat to cat as well as in the corresponding samples from the head and the paw of the same cats. In some samples more than 10^5 viral DNA copies could be detected per GAPDH copy. In other samples there was hardly more than one viral DNA copy per $2.5 \cdot 10^3$ GAPDH copies. The minimal DNA amount needed for the different primer sets to amplify FdPV2 DNA varied considerably from only one DNA copy/µl to more than 10^9 DNA copies/µl. Keeping this difference in sensitivity in mind, the variability of viral DNA load found in our study agrees with another study where infection with FdPV2 was suggested to be limited to focal areas on the skin.[19] Since FdPV2 DNA was detected in normal feline skin before, quantification of the amount of viral DNA was consequently proposed to rule out a possible causative role of FdPV2 in certain types of lesions.[19] In our study we used Cytobrush samples taken directly from BISC lesions as positive controls. However, the amount of FdPV2 DNA in these samples was rather low compared to the samples taken from the normal skin. None of these control samples had more than 200 FdPV2 copies per molecule of GAPDH DNA.

PV infection is often equated to malignant tumour formation. However, asymptomatic infection is more common. Up to 80% of humans are infected asymptomatically with human PV.[18] Various animal species have also been investigated regarding PV infections and asymptomatic PV infections were found regularly. The prevalence varies from species to species. Interestingly, primates showed prevalence rates similar to that of humans.[5] However, no PV DNA could be amplified from samples of the normal skin of cats for a long time. Asymptomatic infection was therefore declined. The 'hit and run' carcinogenesis model was used to explain transformation of normal skin cells into neoplastic cells by transient PV infection.[6] However, a recent study found FdPV2 DNA in skin samples of 39% of healthy cats.[19] In our study, we could detect FdPV2 DNA in at least one Cytobrush sample of 98% of the cats investigated. Cytobrushes absorb cells from the surface of the skin. Subsequently, the cells are lysed in order to extract the DNA for PCR. Therefore, it is not clear if the extracted PV DNA originates from infected epithelium cells or from virions that were attached to the skin's surface. To determine the rate of asymptomatically infected animals only from PCR results might therefore be incorrect. However, it can be stated that 98% of the cats had certainly been in close contact with FdPV2. Considering the high tenacity of PV particles, it makes sense that high amounts of virus are shed to build up an infectious virus reservoir on biological surfaces.

The samples used as negative controls were taken from SPF cats and exact instructions had been given on how to take the samples to ensure sufficient quality for analysis. These cats originated from special SPF catteries and were kept as laboratory animals in facilities with high hygiene standards. Only specifically trained personnel were allowed to enter the SPF facilities and to have contact with the cats. The health status of the cats was checked at regular intervals in order to attest absence of certain pathogens.[29] Originally, they had not specifically been tested for FdPV2. Thus, the fact that FdPV2 is such highly prevalent among normal cats but could not be detected in the samples from those SPF cats, validates the high quality of the SPF facility. Such SPF facilities seem to represent a valuable source for collecting different negative control samples also for other purposes. Using a reference gene in PCR such as GAPDH was important to confirm a sufficient quality of the Cytobrush sample and DNA extraction. Specific FdPV2 DNA could be amplified in qPCR from one sample taken from the paw. However, after quantification the number of FdPV2 copies per GAPDH was within the range of the negative samples.

In our study, we determined the seroprevalence of FdPV2 to be 22% in the investigated population of cats. This was much lower than the DNA prevalence. As expected, the determined seroprevalence is dependent on the applied COV, which were calculated from the OD values of the control sera. The positive control sera were collected from BISC positive cats and the negative control sera from SPF cats. The positive control sera had very high OD values compared to the most sera tested in the analysis. Therefore, the inclusion of these values into the determination of the COV (WPC and MSWC) lead to high COV and consequently to very low

seroprevalence. In contrast, when applying methods to calculate the COV, which are based only on the negative control sera (SNC and XBP2S), the seroprevalences seem to be more reasonable. It is known that PV do not necessarily induce robust antibody responses.[2] Furthermore, the age distribution of the sample population might be an explanation for the low seroprevalence. In our study, 22 out of the 100 tested cats were less than 1 year old. All of them were tested negative in the ELISA. A study in humans showed that it can take many months from the onset of an infection with cutaneous HPV types to seroconversion.[42] It could therefore be that many cats just did not have enough time to produce detectable amounts of antibodies. On the other hand, we do not know how long after seroconversion specific antibodies remain in detectable amounts in the sera. Animals that are now seronegative could have seroconverted before while antibody levels dropped over time. In humans, however, levels of antibodies against HPV were found to be stable even after more than 10 years.[42].

In our study the seropositive cats were significantly older than the seronegative cats. This may fit with the fact that BISC is more common in old cats.[16,17] Nevertheless, the BISC affected cats that served as positive controls showed significantly higher amounts of antibodies than any cat from the sample population. In other species, it was shown that the neutralizing antibodies cannot induce the regression of the PV infection once it is established.[43] The same seems to be true in cats.

The L1 major capsid protein of PV is considered to induce neutralizing antibodies that are mainly type specific.[43] However, two serum samples showed reactions against CPV1 that were as strong as or even stronger than against FdPV2. An ELISA was performed to evaluate the reaction of these samples against CPV1 L1-GST and GST alone as antigens. All of the tested sera reacted against GST alone as strong as against CPV1 L1-GST. GST is an enzyme that plays a key role in cellular detoxification. It is not only present in mammals but also in fungi, helminths and bacteria.[44,45] Therefore it can be assumed that the mentioned five serum samples contained antibodies against GST. Both ELISA antigens, FdPV2 L1 and CPV1 L1, were tagged to GST because GST allows non-denaturing adhesion to coated glutathione-conjugated casein and, thus, formation of virus-like particles as antigen. Alternatively, these two sera might contain antibodies against bacterial proteins that had not been washed away completely after incubating the plates with the antigen. However, we did not expect such reactions since the sera had been pre-incubated with bacterial lysate. In both cases the results would be false positive. Consequently, these sera were not considered positive, neither for FdPV2 nor for CPV1. This shows the importance of a negative control when using a GST capture ELISA.

Comparing the individual cat's results from the ELISA and the qPCR, a certain correlation might be expected. In humans, individuals with a high HPV DNA load are more prone to be also seropositive.[42] Likewise, intimate contact with FdPV2 might as well induce the production of antibodies against it. Therefore, cats with a high virus load are suggested to be more often seropositive. In our study, no such correlation could be found. On the contrary, significantly higher amounts of FdPV2 DNA were detected in seronegative cats. The highest number of copies per GAPDH was 140

times higher than the highest number found in the samples of the seropositive cats. Individuals sharing the same household did not seem to influence each other with regard to either the serological or the PCR outcome. One cat showed a negative paw sample ($3.1 \cdot 10^{-2}$) whereas its partner animal had the highest amount of virus DNA on its paw that was measured in this study ($2.0 \cdot 10^{5}$). From five cats living together on a farm, the head sample from three remained negative while their paw samples were much higher than those from the other two. Still all 15 cats from multiple cat households were tested seronegative.

BISC affected cats served as positive controls. As the Cytobrush samples were taken directly from their lesions, high amounts of FdPV2 DNA could be expected. However, in none of these samples, more than 200 copies of FdPV2 per GAPDH were detected. This was twice the amount of the mean copy number in samples of normal skin of seropositive cats. Still, it was seven times less than the mean copy number detected in normal skin of seronegative cats. Seroconversion and disease development does not seem to be dependent on the virus load alone. This weakens once again the hypothesis of a straightforward role of FdPV2 causing BISC. The virus might as well be a part of the normal skin flora of cats. The immune system would then not produce antibodies against it as long as the skin is intact. The seropositive cats were significantly older than the seronegative. Suggesting that older cats have had more skin traumas in their lives, this might support this theory. However, the two BISC affected cats that served as positive controls in our study showed significantly higher amounts of antibodies against FdPV2 than any of the "healthy" cats. These observations might imply that active and immunogenic replication of FdPV2 precedes the occurrence of BISC, while active replication of the same virus is no longer needed for the progression of the disease. However, more serum samples of BISC affected cats need to be tested.

So far, the causative role of FdPV2 in the development of BISC has only been supported by repeatedly amplifying FdPV2 DNA from BISC samples. In this study, we could detect FdPV2 DNA in normal skin samples of 98% of the investigated cats. This proves that the virus is widespread in the cat population, especially among healthy cats. However, there is nothing known about the virulence of different FdPV2 strains nor its general penetration frequency to clinical illness. The Henle-Koch postulates have not yet been fulfilled and this may also be difficult in the future, since PV cannot be propagated in cell culture to produce a pure stock of inoculating virus. Thus, it is still possible that FdPV2 represents just a simple bystander of another yet unrecognized causative agens. As we digested the skin samples to extract the DNA, we cannot say whether the viral DNA originated from infected skin cells or from virus was lying on the surface of the skin. Using IHC to detect viral proteins in full thickness skin samples might help to determine the origin of the viral DNA. The limits in sensitivity of this technique are described in the beginning of the discussion section. In situ hybridization detects viral DNA in skin samples regardless of the replication status. Samples taken from BISC and normal skin could be compared. In situ hybridization assays using riboprobes might even detect viral RNA and be used to

distinguish if the virus is replicating or not. Difficulties in collecting and processing the samples give this technique its limits in feasibility.
Proving the causative role of a virus in the development of a certain disease is very challenging. In case of FdPV2, the development of BISC lesions must be proven in formerly FdPV2 free cats after infection with the virus. However, this prove is still lacking since FdPV2 cannot be propagated in cell culture on one hand and the virus is widespread in the healthy cat population on the other hand. Collection of different samples (i.e. serum, Cytobrush, full thickness skin biopsies) from the same patient is desirable. Multiple tests can be done and the results can be compared. Moreover, patients should be sampled repeatedly at different points in time to gain information about the interaction of the virus with the cat's body. Studies with large sample population are though needed in order to receive reliable data. This would allow us to obtain a better comprehension of the virus' epidemiology.

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Martin Müller provided the glutathione casein for our ELISA assay.

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Appendix

Study	Primer set	BISC	Viral plaques	SCC	ISCC	OSCC	Other lesions	Normal skin
Kidney et al., 2001 [30]	NO1/NO2						0/50 (0%)	
	$E5^+/E5^-$						0/50 (0%)	
	IFNR-2/IDNT-2						0/50 (0%)	
Antonsson et al., 2002 [5]	FAP59/FAP64							0/5 (0%)
Nespeca et al., 2006 [14]	PapF/PapR	1/21 (5%)			0/22 (0%)		0/11 (0%)	
	CP4/CP5/PPF1	5/21 (24%)			4/22 (18%)		0/11 (0%)	
Munday et al., 2007 [6]	FAP59/FAP64	11/18 (61%)					0/15 (0%)	0/3 (0%)
	IFNR-2/IDNT-2	9/11 (82%)					0/15 (0%)	0/3 (0%)
	[a]	*5/18 (28%)*						
Munday et al., 2008 [18]	[b]	*20/20 (100%)*			*17/20 (85%)*		*3/17 (18%)*	
Munday et al., 2008 [7]	FAP59/FAP64		2/2 (100%)					
	MY09/MY11		2/2 (100%)					
	JMPF/JMPR		*2/2 (100%)*					
Lange et al., 2009 [15]	A16/A37	*3/3 (100%)*						
Munday et al., 2009 [46]	FAP59/FAP64					1/20 (5%)	0/20 (0%)	
	IFNR-2/IDNT-2					0/20 (0%)	0/20 (0%)	
	MY09/MY11					0/20 (0%)	0/20 (0%)	
Munday et al., 2009 [22]	FAP59/FAP64			1/1 (100%)				
	JMPF/JMPR			*1/1 (100%)*				
Anis et al., 2010 [8]	[c]	3/3 (100%)			5/5 (100%)	1/1 (100%)	1/1 (100%)	
	[a]	*3/3 (100%)*			*4/5 (80%)*			
Munday et al., 2010 [19]	MY09/MY11							0/44 (0%)
	JMPF/JMPR							*34/88 (39%)*[d]
Munday et al., 2010 [20]	MY09/MY11		4/14 (29%)				0/14 (0%)	
	FAP59/FAP64		4/14 (29%)				0/14 (0%)	
	JMPF/JMPR		*14/14 (100%)*				*1/14 (7%)*	
Munday et al., 2010 [31]	MY09/MY11						4/7 (57%)	0/120 (0%)
	jmpSA-F/jmpSA-R						6/7 (86%)	0/120 (0%)
Study	Primer set	BISC	Viral plaques	SCC	ISCC	OSCC	Other lesions	Normal skin
Munday et al., 2011 [23]	MY09/MY11			7/70 (10%)				
	JMPF/JMPR			*33/70 (48%)*				
Munday et al., 2011 [24]	[b]	*14/14 (100%)*	*8/8 (100%)*		*12/18 (67%)*		*1/14 (7%)*	

Study	Primer set	BISC	Viral plaques	SCC	ISCC	OSCC	Other lesions	Normal skin
Munday et al., 2011 [47]	FAP59/FAP64					0/30 (0%)		
	MY09/MY11					0/30 (0%)		
O'Neill et al., 2011 [9]	[e]	7/22 (32%)			11/74 (15%)		2/12 (17%)	
	[a]	*4/22 (18%)*			*4/74 (5%)*			
Schwittlick et al., 2011 [48]	[b]	*1/1 (100%)*						
Munday et al., 2013 [3]	FAP59/FAP64	0/1 (0%)						
	JMPF/JMPR	0/1 (0%)						
	MY09/MY11	1/1 (100%)						
	JMY2F/JMY2R	1/1 (100%)						
Dunowska et al., 2014 [13]	FAP59/FAP64						0/1 (0%)	
	MY09/MY11						1/1 (100%)	
	Unnamed set						1/1 (100%)	

Table 6. Rates of reported PV DNA findings in skin samples from cats using PCR. FdPV2 DNA findings are in italic.

BISC = Bowenoid in situ carcinoma, SCC = squamous cell carcinoma, ISCC = infiltrative squamous cell carcinoma (when defined in study), other lesions = actinic keratosis, allergic dermatitis, apocrine gland cyst, apocrine gland cystadenoma, dermatophytosis, dysplasia, eosinophilic granuloma, eosinophilic plaques, feline leprosy, fibrosarcoma, glossitis, granulomatous dermatitis, hyperplastic tonsil, hypersensitivity dermatitis, mast cell tumour, melanoma, periodontal disease, plasmacytic stomatitis, sarcoids, trichoblastoma, ulcerative gingivitis.

[a] sequences later identified by Lange et al. [15], [b] nested PCR with FAP59/FAP64 and JMPF/JMPR, [c] HPV1, HPV2, HPV4, HPV7, HPV8, HPV10, P1, P6, P8, [d] equals 23/44 cats (52%), [e] FAP59/FAP64 and nested PCR with CP65/CP70 and CP66/CP69

No.	Breed	Sex	Age[1]	ELISA		PCR head		PCR paw		PCR cat[3]
				OD	Result	Copies[2]	Result	Copies[2]	Result	
1	Mixed breed	Fc	6.9	0.2443	neg.	0.0887	neg.	0.1632	neg.	neg.
2	Mixed breed	Mc	6.9	0.1839	neg.	0.0664	neg.	0.1184	neg.	neg.
3	Mixed breed	Mc	1.3	0.1854	neg.	0.0117	neg.	0.1880	neg.	neg.
4	Mixed breed	Mc	5.0	0.2916	neg.	0.0443	neg.	0.0002	neg.	neg.
5	Mixed breed	Mc	17.6	0.2198	neg.	0.0443	neg.	0.0664	neg.	neg.

Table 7. Overview of individual data and test results of the negative control cats

Fc = female castrated, Mc = male castrated

[1] in years, [2] FdPV2 per GAPDH, [3] defined as positive when at least 1 PCR sample positive

No.	Breed	Sex	Age[1]	ELISA		PCR sample 1		PCR sample 2		PCR cat[3]
				OD	Result	Copies[2]	Result	Copies[2]	Result	
1	Maine Coon	Fc	12.8	2.0406	pos.	165.7712	pos.	13.2511	pos.	pos.
2	Mixed breed	Fc	17.0	1.7428	pos.	3.7526	pos.	n/a	n/a	pos.

Table 8. Overview of individual data and test results of the positive control cats

Fc = female castrated

[1] in years, [2] FdPV2 per GAPDH, [3] defined as positive when at least 1 PCR sample positive

No.	Breed	Sex	Age[1]	ELISA		PCR head		PCR paw		PCR cat[3]
				OD	Result	Copies[2]	Result	Copies[2]	Result	
1	Mixed Breed	F	0.8	0.1868	neg.	58.1412	pos.	1.0550	pos.	pos.
2	British Shorthair	M	0.5	0.1962	neg.	5.4501	pos.	8.5881	pos.	pos.
3	Mixed Breed	F	10.0	0.1899	neg.	46.6417	pos.	38.3712	pos.	pos.
4	Mixed Breed	Mc	7.8	0.2183	neg.	78.5387	pos.	61.4105[5]	pos.	pos.
5	Mixed Breed	F	0.8	0.1826	neg.	22.5761	pos.	147.1966	pos.	pos.
6	Persian	F	12.1	0.5646	pos.	44.9197	pos.	22.5936	pos.	pos.
7	Mixed Breed	Mc	3.0	0.2466	neg.	3.2017	pos.	89.1296	pos.	pos.
8	Mixed Breed	Mc	11.8	0.7209	pos.	49.3410	pos.	10.7638	pos.	pos.
9	Ocicat	Mc	0.9	0.2109	neg.	1.3704	pos.	9.9065	pos.	pos.
10	Persian	Fc	1.0	0.2193	neg.	3.5387	pos.	1416.9286[5]	pos.	pos.
11	Mixed Breed	Fc	13.8	0.5320	pos.	7.0872	pos.	26.1431[5]	pos.	pos.
12	Mixed Breed	Fc	14.0	0.2524	neg.	2.7994	pos.	9.0710	pos.	pos.
13	Bengal	F	0.4	0.2659	neg.	2.0334	pos.	4.5716	pos.	pos.
14	Bengal	F	0.5	0.2634	neg.	0.7160	pos.	2.7002	pos.	pos.
15	Mixed Breed	F	n/a	0.2499	neg.	76.1697	pos.	6.7338	pos.	pos.
16	Mixed Breed	M	n/a	0.2130	neg.	1.2656	pos.	5.4713	pos.	pos.
17	Mixed Breed	F	n/a	0.2757	neg.	0.1004	neg.	107.0025	pos.	pos.
18	Mixed Breed	F	n/a	0.2314	neg.	0.0555	neg.	20.5851	pos.	pos.
19	Siamese	Fc	13.1	0.2782	neg.	15.8124	pos.	2461.2182	pos.	pos.
20	Mixed Breed	F	n/a	0.2302	neg.	0.0023	neg.	29.0754	pos.	pos.
21	Mixed Breed	Mc	2.6	0.2462	neg.	4.8777	pos.	3.0359	pos.	pos.
22	Mixed Breed	Mc	12.0	0.7177	pos.	70.0246	pos.	280.3176	pos.	pos.
23	Mixed Breed	M	0.5	0.2113	neg.	12.2588	pos.	10.4068	pos.	pos.
24	Mixed Breed	Fc	13.0	0.2255	neg.	0.5141	pos.	2.9148	pos.	pos.
25	Mixed Breed	Fc	0.8	0.2036	neg.	47.8899	pos.	33.5388	pos.	pos.
26	Mixed Breed	Fc	1.5	0.2376	neg.	21.2677	pos.	19.7329	pos.	pos.
27	Mixed Breed	Mc	8.5	0.2244	neg.	1.8758	pos.	3.6419	pos.	pos.
28	Mixed Breed	M	0.6	0.2299	neg.	4.3960	pos.	5.1480	pos.	pos.
29	Mixed Breed	F	0.6	0.2387	neg.	4.8891	pos.	3.0218	pos.	pos.
30	Mixed Breed	M	1.1	0.2365	neg.	3.2924	pos.	374.5652	pos.	pos.
31	Mixed Breed	F	0.5	0.3131	neg.	0.0009	neg.	9.7538	pos.	pos.
32	Mixed Breed	Fc	4.6	0.2255	neg.	0.1434	neg.	0.5673	pos.	pos.

No.	Breed	Sex	Age[1]	ELISA		PCR head		PCR paw		PCR cat[3]
				OD	Result	Copies[2]	Result	Copies[2]	Result	
33	Siberian	Fc	7.8	0.2529	neg.	2.6556	pos.	5.6988	pos.	pos.
34	NFC x Persian	Mc	7.0	0.2469	neg.	0.0396	neg.	1.4497[5]	pos.	pos.
35	Mixed Breed	M	0.7	0.1928	neg.	133.1180[6]	n/a	0.3704	pos.	pos.
36	Mixed Breed	M	0.3	0.3157[4]	neg.	2.4430	pos.	0.0590	neg.	pos.
37	Mixed Breed	F	0.8	1.0938[4]	neg.	18.9307	pos.	56.8713	pos.	pos.
38	Mixed Breed	F	0.8	0.2272	neg.	60.8203	pos.	251.5452[5]	pos.	pos.
39	Mixed Breed	Mc	3.0	0.2449	neg.	378.3665	pos.	125.3611	pos.	pos.
40	Burmese	Mc	13.0	0.4515	pos.	4.5468	pos.	7.7520	pos.	pos.
41	Maine Coon	F	0.4	0.5223[4]	neg.	5.8695	pos.	22.4636	pos.	pos.
42	Mixed Breed	Mc	2.8	0.3029	neg.	2.6556	pos.	4.1412	pos.	pos.
43	Mixed Breed	Fc	11.0	0.2666	neg.	45.3404	pos.	6.9757	pos.	pos.
44	Ragdoll	Mc	10.8	0.2091	neg.	2.9238[5]	pos.	1.5408	pos.	pos.
45	Mixed Breed	Fc	12.0	0.4538	pos.	59.4657	pos.	71.5011	pos.	pos.
46	NFC	M	3.8	0.1898	neg.	17.7842	pos.	1.0824	pos.	pos.
47	Birman	M	14.0	0.2120	neg.	0.5642	pos.	4.8362	pos.	pos.
48	Mixed Breed	Mc	9.7	0.6321	pos.	0.7166	pos.	0.6067	pos.	pos.
49	Mixed Breed	Mc	10.0	0.2043	neg.	1.0674	pos.	3.8258	pos.	pos.
50	Mixed Breed	Mc	17.0	0.1763	neg.	0.4111	pos.	8.8936	pos.	pos.
51	Mixed Breed	Mc	4.1	0.2149	neg.	0.0055	neg.	1.7003[5]	pos.	pos.
52	Mixed Breed	Fc	13.0	0.3498[4]	neg.	27.8191	pos.	418.5231	pos.	pos.
53	Mixed Breed	Fc	3.9	0.1975	neg.	391.9568	pos.	39.5821	pos.	pos.
54	Mixed Breed	Mc	14.0	0.2544	neg.	620.5154	pos.	54.6866	pos.	pos.
55	Mixed Breed	Mc	11.0	0.1780	neg.	2.6495	pos.	0.7194	pos.	pos.
56	Persian	F	14.0	0.9130	pos.	1049.3972	pos.	6.3803	pos.	pos.
57	Mixed Breed	Fc	11.0	0.2071	neg.	0.2209	pos.	0.8372	pos.	pos.
58	Mixed Breed	Mc	3.0	0.1807	neg.	1289.9630	pos.	22.0658	pos.	pos.
59	Mixed Breed	Mc	7.0	0.4153	pos.	0.6027	pos.	3.8437	pos.	pos.
60	Burmese	Mc	8.1	0.2663	neg.	1.4340	pos.	8.5948	pos.	pos.
61	Mixed Breed	Mc	9.0	0.6255	pos.	0.8576	pos.	0.4428	pos.	pos.
62	Mixed Breed	Mc	14.9	0.2018	neg.	0.3727	pos.	16.9218	pos.	pos.
63	Mixed Breed	F	1.9	0.1881	neg.	9368.4824	pos.	17860.9598	pos.	pos.
64	Mixed Breed	Mc	8.0	0.4036	pos.	1.5492	pos.	4.2337	pos.	pos.

No.	Breed	Sex	Age[1]	ELISA		PCR head		PCR paw		PCR cat[3]
				OD	Result	Copies[2]	Result	Copies[2]	Result	
65	Mixed Breed	Mc	12.3	0.6086	pos.	90.9880	pos.	181.7245	pos.	pos.
66	Mixed Breed	F	1.1	0.2198	neg.	3.2141	pos.	14.5063	pos.	pos.
67	Mixed Breed	M	0.8	0.2219	neg.	1.6199	pos.	14.3653[6]	n/a	pos.
68	Mixed Breed	Mc	n/a	0.8141	pos.	207.8381	pos.	1462.7781	pos.	pos.
69	Mixed Breed	Fc	7.0	0.2133	neg.	11.4578[5]	pos.	32.2112	pos.	pos.
70	British Shorthair	Mc	4.1	0.2522	neg.	6.8150	pos.	4.0253	pos.	pos.
71	Mixed Breed	Mc	16.8	0.3075	neg.	84.5158	pos.	1.3934	pos.	pos.
72	Mixed Breed	Mc	12.0	0.2046	neg.	9.8834	pos.	10.9834	pos.	pos.
73	Mixed Breed	Mc	0.7	0.2154	neg.	20.6652	pos.	0.2316	pos.	pos.
74	Mixed Breed	M	n/a	0.2014	neg.	44.2468	pos.	8.2811	pos.	pos.
75	Mixed Breed	Mc	3.9	0.2544	neg.	4.4234	pos.	4.2384	pos.	pos.
76	Mixed Breed	Mc	n/a	0.2436	neg.	0.0320	neg.	3.5802	pos.	pos.
77	Egyptian Mau	Fc	8.2	0.5305	pos.	0.3929	pos.	9.0710	pos.	pos.
78	Mixed Breed	Mc	17.0	0.1956	neg.	23.8190	pos.	26.2857	pos.	pos.
79	Mixed Breed	M	0.7	0.1991	neg.	21.1195	pos.	0.0313	neg.	pos.
80	Mixed Breed	M	0.7	0.2017	neg.	29.8767	pos.	202664.0048	pos.	pos.
81	British Shorthair	Mc	8.0	0.4730	pos.	2.8990	pos.	5.5140	pos.	pos.
82	Siberian	F	6.3	0.3234[4]	neg.	132.8222	pos.	157.7321	pos.	pos.
83	Mixed Breed	Mc	10.1	0.2095	neg.	16.2059	pos.	3.9656	pos.	pos.
84	Mixed Breed	Mc	9.3	0.1939	neg.	0.2246	pos.	2.5905	pos.	pos.
85	Maine Coon	Mc	12.3	0.3399	pos.	0.0004	neg.	12.2418	pos.	pos.
86	Mixed Breed	Mc	10.1	0.1565	neg.	0.0380	neg.	1.0258	pos.	pos.
87	Mixed Breed	Mc	5.1	0.1591	neg.	0.4531	pos.	5.0756	pos.	pos.
88	Mixed Breed	M	n/a	0.4017	pos.	135.5546	pos.	27.8022	pos.	pos.
89	Mixed Breed	Mc	n/a	0.4039	pos.	0.2997	pos.	14.3653[6]	n/a	pos.
90	Mixed Breed	Mc	7.0	0.1911	neg.	0.0034	neg.	0.2150	pos.	pos.
91	Mixed Breed	Mc	13.0	0.2726	neg.	34.7049	pos.	1.2834	pos.	pos.
92	Mixed Breed	Fc	4.8	0.1929	neg.	9.7235	pos.	0.0272	neg.	pos.
92	Mixed Breed	Mc	9.8	0.1820	neg.	3.9441	pos.	0.0059	neg.	pos.
94	Mixed Breed	Fc	9.1	0.2363	neg.	61.5809	pos.	4.3892	pos.	pos.
95	Siamese	Fc	14.2	0.2644	neg.	0.0099	neg.	0.0138	neg.	neg.
96	Persian	M	4.6	0.2626	neg.	0.3797	pos.	0.6535	pos.	pos.

No.	Breed	Sex	Age[1]	ELISA		PCR head		PCR paw		PCR cat[3]
				OD	Result	Copies[2]	Result	Copies[2]	Result	
97	Mixed Breed	F	0.3	0.1539	neg.	0.1094	neg.	0.0893	neg.	neg.
98	Turkish Van	M	0.4	0.1539	neg.	1.1300	pos.	1.6728	pos.	pos.
99	Mixed Breed	Fc	15.3	0.6067	pos.	8.5867	pos.	0.0238[5]	neg.	pos.
100	Mixed Breed	Mc	11.8	0.2233	neg.	0.3238	pos.	0.2740	pos.	pos.

Table 9. Overview of individual data and test results of the sample population

NFC = Norwegian forest cat, F = female, Fc = female castrated, M = male, Mc = male castrated

[1] in years, [2] FdPV2 per GAPDH, [3] defined as positive when at least 1 PCR sample positive, [4] reaction against GST (see discussion), [5] 2 products after amplification (see results), [6] negative for GAPDH (see results)

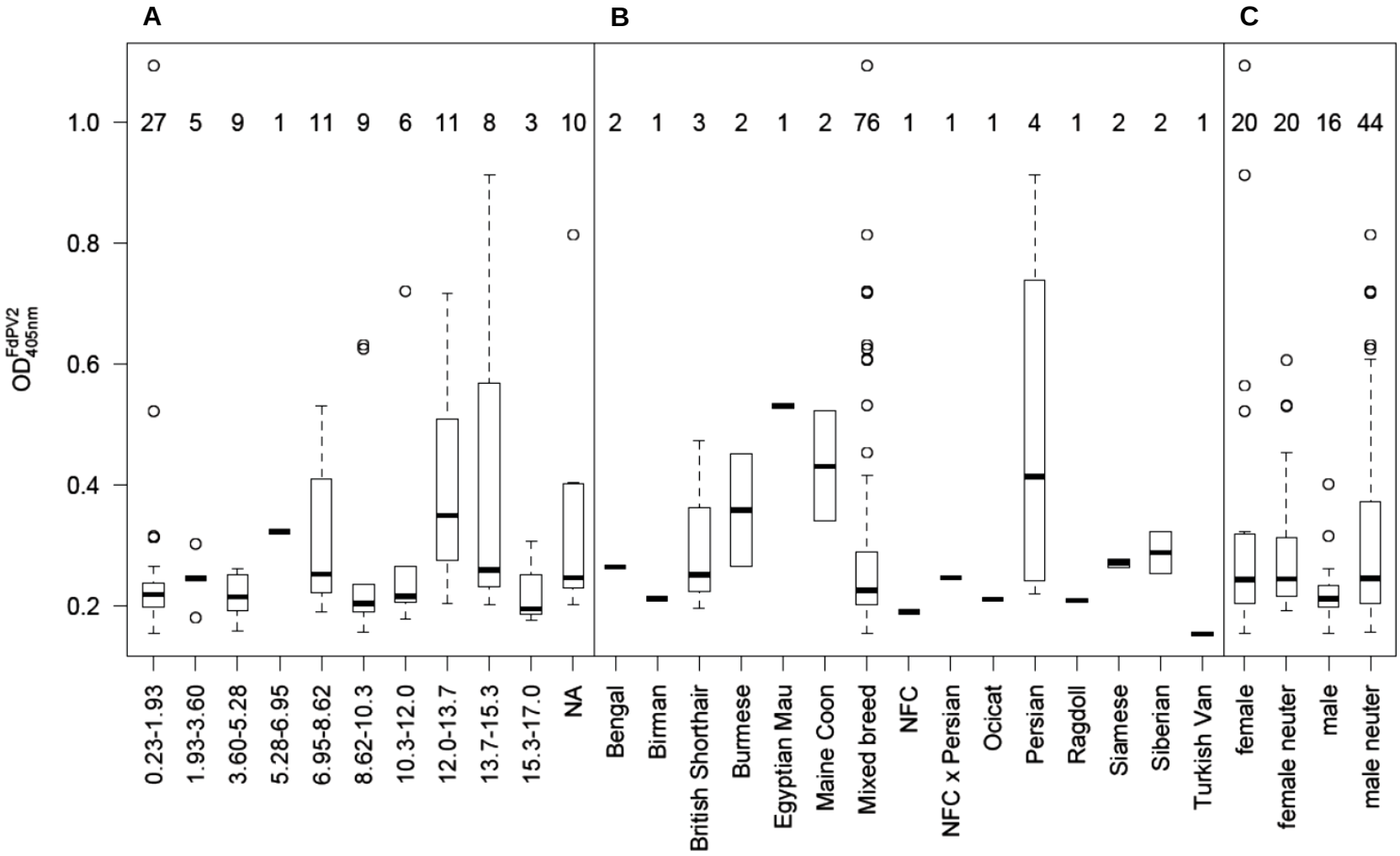

Figure 6. Boxplots of FdPV2 ELISA OD values from cats divided into subsets based on age **(A)**, breed **(B)** or sex **(C)**. For the definition of the age groups, cats were divided into 10 categories each spanning approximately 1.7 years. The categories for sex were abbreviated as F, Fc, M and Mc for female, female castrated, male and male castrated, respectively. The numbers of the individual group sizes are indicated on top of the graphs.